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Correlation of survival rates of *Anopheles dirus* A (Diptera: Culicidae) with different infection densities of *Plasmodium cynomolgi**

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The survival rates are described for 36 paired replicates of Anopheles dirus *A mosquitos that had been allowed to engorge themselves on rhesus monkeys that were either infected or non-infected with* Plasmodium cynomolgi. *The survival rates of infected mosquitos with a mean number of oocysts less than 10 did not differ significantly from those that were non-infected; however, there was a significant difference in the survival rates of non-infected groups and those with a mean number of oocysts in the range 10–40, 41–70, or* $\geqslant 71$.

Daily survival rates for non-infected and infected mosquitos did not differ significantly during the first 8 days of extrinsic incubation. In contrast, for the period 9–30 days the survival rates of mosquitos with mean number of oocysts $\geqslant 41$ *were significantly different from those of non-infected mosquitos. The cumulative daily survival rates of mosquitos with mean number of oocysts up to 40, 41–70, or* $\geqslant 71$ *decreased with the oocyst count. Mosquitos with a mean number of oocysts* $\geqslant 71$ *frequently exhibited excessive numbers of bacteria and deterioration of both their guts and salivary glands.*

Various assumptions have been made about certain entomological aspects of the epidemiology of human malaria. For example, in defining certain entomological parameters, Macdonald (*1*), Garrett-Jones (*2*), and Molineaux (*3*) assumed that the longevity and daily survival rates of infected mosquitos are unaffected by malarial parasites. However, recent studies, including some results we have reported (*4*), refute these basic assumptions and indicate, *inter alia*, that the malarial parasite has a detrimental effect on mosquito survival rate (*5*). Klein et al. (*4*) as well as Gad et al. (*6*) have shown that the mortality rate is significantly higher for infected than for non-infected mosquitos. Also, Hacker (*5*) reported a significant reduction in the fecundity of mosquitos infected with malarial parasites. Furthermore, Schiefer et al. (*7*) demonstrated that the degree of infection of *Anopheles stephensi* Liston with *Plasmodium cynomolgi* Mayer correlates negatively with the mosquitos' flight performance.

Here, we report the results of a study to determine the influence of *Plasmodium cynomolgi* infections of *Anopheles dirus* A, a known primary vector of human malaria parasites in Thailand (*8*) and of simian malaria parasites in southeast Asia (*7–10*), on the mosquito's daily survival rate and the degree of correlation between survival rate and degree of infection.

MATERIALS AND METHODS

The B strain of *P. cynomolgi* (*P.c. bastianellii*) and *A. dirus* A were used, as previously described (*4*). In the study design (Fig. 1) two separate batches of adult female mosquitos were allowed to feed simultaneously on two rhesus monkeys: one non-infected and the other infected with *P. cynomolgi*. The first batch, consisting of 40 adult females, fed on a non-infected rhesus monkey for approximately 30 minutes. Twenty of the engorged females, who served as non-infected controls, were then placed in a screened cardboard specimen cup and provided with

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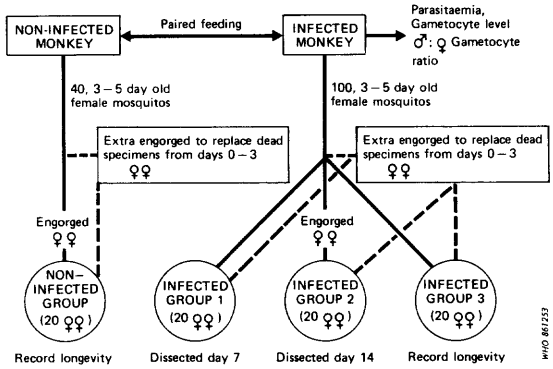

Fig. 1. Schematic representation of the sequence of events for each replicate of mosquitos.

a 5% solution of multivitamin syrup for the remainder of the study, while the remaining 20 engorged females were held in a reserve cage. During the first 3 days of the study, dead or moribund mosquitos in the non-infected group were noted and replaced with others from the reserve cage. Mosquitos still remaining in the reserve cage after 3 days were discarded, while dead mosquitos among the controls were removed daily, counted, and those data used to calculate survival rates.

The mosquitos in the second batch, consisting of 100 adult females, were allowed to feed for approximately 30 minutes on a rhesus monkey infected with *P. cynomolgi*. Sixty of the engorged females were then divided into three groups of 20, placed in separate specimen cups (infected groups 1, 2, and 3), and provided with a 5% multivitamin solution for the rest of the study. The remaining engorged females were held in a cage until the third day of the study and then discarded. Mosquitos in each of the infected groups that died during the first 3 days of the study were replaced with others from the reserve infected cage, and the day of death was recorded.

Mosquitos from infected group 1 were dissected on the seventh day of the study, their midguts removed, and the number of oocysts counted. The mean diameter of five oocysts from each of five mosquitos was also determined, and the diameter of the largest and smallest oocysts used as an estimate of the size range. Mosquitos that died prior to the 7th day of the study were also dissected and the number of oocysts counted.

Mosquitos in infected group 2 that were still alive on the 14th day of the study were dissected and their midguts examined for oocysts and remnants of burst oocysts. Their salivary glands were removed, and the sporozoite rate was determined. The general condition of the salivary glands and midgut, e.g., ruptured or discoloured, was also noted. Mosquitos that died prior to the 14th day were also dissected and, when possible, the number of oocysts was estimated and the sporozoite rate determined.

Mosquitos in infected group 3 were observed at least twice daily for signs of morbidity and mortality. After the third day of extrinsic incubation all dead or moribund mosquitos in this group were dissected immediately or preserved, as described by Ward (*11*), and held for later dissection. The midguts of mosquitos that died on days 3–8 were dissected and an estimate was made of the number of oocysts and their stage of development. Both midguts and salivary glands of mosquitos that died on or after day 9 were dissected and an estimate was made of the number of oocysts and the sporozoite rate; the condition of the salivary glands and midgut was also noted.

The number of dead mosquitos was recorded daily throughout the study, and the range and mean survival rates were calculated for each replicate. No accurate count of the number of oocysts for the dead mosquitos in group 3 could be made because many either had ruptured oocysts or midguts that were partially or totally decomposed; the mean number of oocysts for infected group 1 was therefore taken as an estimate for that of group 3 (see Annex 1). Examination of those mosquitos in group 3 in which mature and ruptured oocysts could be counted indicated that the value did not differ significantly from the mean number of oocysts for group 1.

The temperature throughout the study ranged from 22.2 °C to 32.2 °C for both the non-infected and infected groups of mosquitos. The mean temperature for infected groups was 26.0 ± 0.6 °C, while that for the non-infected groups was 26.6 ± 0.8 °C.

RESULTS

A total of 36 paired replicate batches of adult female mosquitos were fed on infected or non-infected rhesus monkeys from 9 August 1979 to 25 March 1980, and 2160 were subsequently dissected. The mean oocyst diameter for all infected groups was as expected, and the mean number of oocysts per mosquito for different replicates (calculated using data from group 1) ranged from 0.05, i.e., 1 oocyst per 20 mosquitos, to 169.8, with an average of 48.4 for all replicates. The proportion of infected mosquitos for the 36 replicates of groups 1, 2, and 3 ranged from 5% to 100%, while the mean proportion of infected mosquitos was 78.8%, 76.3%, and 66.1%,

Table 1. Effect of the mean number of oocysts on the mean survival of mosquitos in the study

Status of mosquitos	Mean number of oocysts (M)	No. of paired replicates	Mean survival (days)	Student's t-test[a]
Non-infected	–	7	40.5 (35.1–49.3)[b]	0.487
Infected	<10		39.5 (33.4–48.6)	
Non-infected	–	9	42.0 (35.7–51.3)	2.810[c]
Infected	10–40		38.1 (30.6–45.8)	
Non-infected	–	12	37.7 (22.0–49.9)	4.883[d]
Infected	41–70		29.8 (25.3–37.0)	
Non-infected	–	8	41.8 (29.5–54.8)	5.696[d]
Infected	⩾71		24.0 (15.7–40.2)	

[a] Test was applied to the mean survival times of paired non-infected and infected mosquitos.
[b] Figures in parentheses are the range.
[c] $P<0.05$.
[d] $P<0.01$.

respectively.[a] Differences between these proportions were not statistically significant ($F_{2,105}=2.0403$).

The validity of using the number of oocysts in group 1 as an estimate of the number in group 3 was assessed by feeding three separate batches of mosquitos simultaneously on a rhesus monkey infected with *P. cynomolgi*. Each triplicate feeding was replicated nine times, and the number of oocysts per mosquito determined. The within-replicate variance for number of oocysts was 146.78 (18 degrees of freedom), while the among-replicate variance was 2077.6 (8 degrees of freedom); the intraclass correlation (r_1) (*12*), which can be estimated using the following equation,

$$r_1=(s^2_A-s^2_W)/(s^2_A-2s^2_W)$$

is +0.814, which is reasonably high. The within-replicate variance determined was also used to find unbiased estimates of regressions on the number of oocysts (see Annex 1).

The regression of the mean survival rates of infected mosquitos (group 3) on the mean number of oocysts per mosquito (group 1) was negative (slope = −0.16)[b] and differed significantly from zero ($P<0.01$). The intercept on the ordinate, which provides an estimate of the mean longevity of non-infected mosquitos, was 40.5 days, almost the same as that for the non-infected groups (40.2 days). The correlation coefficient (r) of the mean survival rates on the mean number of oocysts per mosquito was −0.74, while the regression of the differences in the mean survival rates of the non-infected and infected mosquitos on the mean number of oocysts per mosquito was positive (slope = +0.18)[c] and significantly different from zero ($P<0.01$). The correlation coefficient was +0.80.

The variation in mean survival rates of the non-infected and infected groups is shown in Table 1. For a mean number of oocysts (M) less than 10 there was no significant difference between the mean survival rates of the non-infected and the infected groups; however, for M greater than 10 the mean survival rates for infected groups were significantly different from those of the non-infected. Furthermore, both the non-infected and infected mortality rates increased with the age of the mosquitos, corroborating the findings reported by Clements & Paterson (*13*).

The cumulative frequency distributions of the non-infected and corresponding infected sample groups were computed for the Kolmogorov-Smirnov 2 sample tests (*14*). Comparison of the daily cumulative survival rates of the paired non-infected and infected groups with different mean levels of infectivity indicates that for $M\leqslant 40$ there was no significant difference between infected and non-infected groups; however, for $M\geqslant 71$ the difference was significant ($P<0.01$). Also, the infected group with $M=41$–70 differed from the non-infected group at the $P<0.10$ level. The number of days required for the death of 50% of the sample (LT_{50}) of the paired non-infected groups and infected groups with $M\leqslant 40$, 41–70, and $\geqslant 71$ were 42.5, 40.5; 40.5, 30.5; and 43.5, 17.0 days, respectively. The cumulative

[a] Data from mosquitos in group 3 that were dead or whose guts and salivary glands were infiltrated with bacteria and/or protozoans were not included in the study.
[b] Slope = −0.19 when corrected for bias (see Annex 1).
[c] Slope = +0.21 when corrected for bias (see Annex 1).

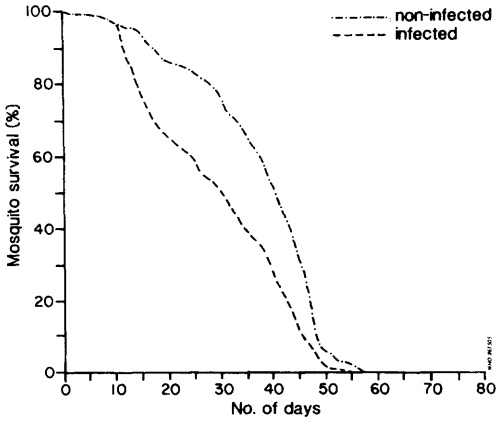

Fig. 2. Cumulative daily survival rate of non-infected mosquitos and of mosquitos infected with mean number of oocysts in the range 41–70.

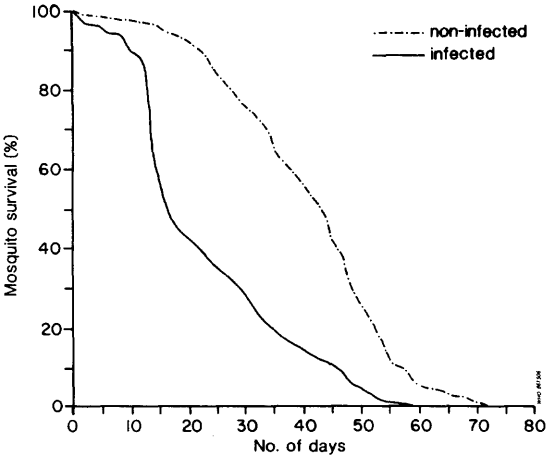

Fig. 3. Cumulative daily survival rate of non-infected mosquitos and of mosquitos infected with mean number of oocysts >71.

survival rates for days 0–9 of the study for all sample groups were the same, while those of the non-infected and of the infected groups with $M \leqslant 40$ were quite similar; however, the cumulative rates of non-infected groups and infected groups with $M = 41$–70 (Fig. 2) and $M \geqslant 71$ (Fig. 3) were different, the greatest decrease in survival rates being in infected groups for days 9–18 of the study. The lowest mortality rate was among mosquitos with $M \leqslant 40$, while the greatest was among those with $M \geqslant 71$. Approximately 42% of all specimens with $M \geqslant 41$ were dead by day 20 of the study. This may correspond to the total number of mosquitos in groups with $M \geqslant 41$ that were infected with more than 100 oocysts.

The daily survival rates in non-infected and infected groups for days 0–3 and 4–8 of the study were not significantly different (Table 2). For days 9–13, 14–20, and 21–30, however, there was a

Table 2. Differences in the mortality rates of the non-infected and infected mosquitos in the study

	Mean mortality (%)					
	Paired replicates (16)		Paired replicates (20)		Total paired replicates (36)	
Days	Non-infected	Infected[a]	Non-infected	Infected[b]	Non-infected	Infected
0–3	2.03	0.60	1.46	2.20	1.72	1.49
4–8	0.31	1.24	1.00	1.02	0.69	1.11
9–13	0.31	1.25	1.53	11.07[c]	0.98	6.70[c]
14–20	3.54	2.86	8.11	29.97[c]	6.08	17.93[c]
21–30	10.14	18.38	15.10	31.33[c]	12.89	25.57[c]
31–40	34.31	35.82	31.96	35.68	33.01	41.30

[a] Mean number of oocysts $\leqslant 40$.
[b] Mean number of oocysts $\geqslant 41$.
[c] Significant at the $P = 0.01$ level.

significant difference between the daily survival rates of non-infected groups and of infected groups with $M \geqslant 41$. For days 31–40 there was no significant difference in the daily survival rates of the non-infected and infected groups, although the cumulative survival rate was much less for infected groups with $M \geqslant 41$.

Regression analysis of the gametocyte number and gametocyte sex ratio, determined when the mosquitos were fed, indicated that there was no relationship between these parameters and the mean number of oocysts.

DISCUSSION

The results we have reported here show that decreases in the survival rate of mosquitos infected with *P. cynomolgi* correlated strongly with increases in the number of oocysts in the gut. The survival rate of mosquitos decreased by 0.166 days per oocyst in the gut, while the difference in the survival rates of non-infected and infected mosquitos increased by 0.187 days per oocyst. Both regressions were very highly significantly different from zero.

On average, the survival rates of mosquitos with $M < 10$ were not significantly different from those of non-infected mosquitos. In contrast, the mean survival rates of the infected groups with $M \geqslant 41$ were significantly different from the non-infected groups for days 9–13, 14–20, and 21–30. After 30 days, there was no significant difference in the mean survival rate of the two groups. This, nevertheless, may reflect the level of infectivity of mosquitos that survived until this time; mosquitos that were heavily infected having died and those with only light or no infection having survived and resumed the normal characteristics of non-infected mosquitos.

It therefore appears that a small number of oocysts has little or no detrimental effect upon an infected mosquito population. For example, for values of M per infected mosquito less than 10, as many as 19 out of 20 of the mosquitos had no oocysts or sporozoites. In such a situation there are statistically too few infected mosquitos to alter the survival rate of the group as a whole. However, the proportion of infected mosquitos increased with the mean number of oocysts per infected mosquito. The number of oocysts in dead mosquitos, especially after the oocysts had begun to rupture, could not be determined accurately. Correlation between groups of the detrimental effects of numbers of oocysts is then difficult. The mean number of oocysts estimated from a sample population of infected laboratory mosquitos might, nevertheless, be useful in predicting the survival rate of infected wild populations, provided environmental stress factors are also taken into consideration.

Over an 8-day period after the mosquitos had engorged on an infected monkey, there was no difference in their cumulative survival rate. However, the survival rate of infected mosquitos decreased after day 9, especially those from "heavily" infected groups, and it can be concluded that the probability of a mosquito dying was directly related to the level of infection with oocysts. By day 20 approximately 42% of mosquitos in the infected groups with $M \geqslant 41$ were dead, and this proportion probably corresponds to the fraction of infected individuals with more than 100 oocysts.

The results of dissection of the salivary gland and midgut of infected mosquitos on day 14 and of dead specimens of infected mosquitos on days 9–20 also indicate that the survival rate was inversely proportional to the infection density. Abnormalities were observed upon dissection of a large proportion of mosquitos in groups with $M \geqslant 71$: in many instances, the midgut and other abdominal organs were almost completely decomposed and infiltrated by bacteria. The salivary glands of heavily infected, live mosquitos apparently ruptured prior to or during dissection and were often black-grey to green and partially decomposed with numerous bacteria. In several cases, all that remained of the glands were the salivary ducts. These findings, in conjunction with the observation that the proportion of infected mosquitos with more than 100 oocysts roughly corresponds to the fraction that died by day 20 of the study, strongly suggest that massive rupturing of a large number of oocysts and the subsequent migration of sporozoites to the salivary glands considerably reduced the survival rate of infected mosquitos. In contrast, light infections only slightly decreased the survival rate of mosquitos, perhaps by increasing their susceptibility to environmental stress.

The results we have reported here indicate that, under laboratory conditions, there is a high correlation between the survival rate of *A. dirus* A mosquitos and the degree of infection with *P. cynomolgi*. The midguts of wild-caught mosquitos usually exhibit a few oocysts, and this corroborates our findings that mosquitos with large numbers of oocysts died as a result of infection as well as environmental factors. In nature, where individuals are subjected to greater levels of environmental stress, this effect may be more pronounced than in the laboratory. A reduced vector survival rate has significant epidemiological implications since it decreases the number of oviposition cycles.

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RÉSUMÉ

CORRELATION ENTRE LES TAUX DE SURVIE D'*ANOPHELES DIRUS* A (DIPTERES: CULICIDAE) ET LES TAUX D'INFESTATION PAR *PLASMODIUM CYNOMOLGI*

Trente-six lots appariés identiques d'*Anopheles dirus* A, vecteur du paludisme confirmé chez l'homme et présumé chez le singe, ont été nourris sur deux singes rhésus, le premier indemne, le second infesté par *Plasmodium cynomolgi*. Vingt moustiques gorgés de sang constituaient le groupe témoin non infesté, et 60 autres moustiques infestés ont été divisés en trois groupes de 20 moustiques (groupes infestés 1, 2 et 3). On a évalué le nombre moyen d'oocystes portés par les moustiques en disséquant ceux du groupe 1 au septième jour de l'étude; on a recherché la présence de sporozoïtes dans les glandes salivaires, et noté l'aspect général de ces dernières et de l'intestin, en disséquant les moustiques du groupe 2 au 14^{e} jour; les taux de survie journaliers ont été calculés à partir des données recueillies sur les moustiques du groupe 3. Les taux d'infestation (c'est-à-dire le nombre moyen d'oocystes) utilisés dans le calcul avec les taux de survie journaliers (groupe infesté 3) étaient ceux du groupe infesté 1, car on n'a pas pu déterminer avec exactitude le nombre d'oocystes portés par les moustiques du groupe 3.

On a établi les courbes de régression entre: 1) le taux de survie moyen des groupes infestés et le nombre moyen d'oocystes par moustique et 2) la différence de taux de survie entre groupes non infestés et infestés et le nombre moyen d'oocystes par moustique. Les taux de survie moyens des groupes infestés présentaient une corrélation négative ($r = -0,74$) avec le nombre moyen d'oocystes; par contre, en ce qui concerne la différence entre les taux de survie moyens des groupes non infestés et infestés la corrélation avec le nombre moyen d'oocystes était positive ($r = +0,80$). En moyenne, le taux de survie des moustiques infestés diminuait de 0,166 jour par oocyste. Les taux de survie des *A. dirus* A portant un nombre moyen d'oocystes inférieur à 10 n'étaient pas significativement différents de ceux des groupes non infestés; par contre, les taux de survie moyens des moustiques non infestés et des moustiques infestés ayant un nombre moyen d'oocystes de 10–40, 41–70 ou $\geqslant 71$, présentaient une différence significative. Il semble par conséquent que la présence de quelques oocystes n'ait aucun effet néfaste sur la survie des *A. dirus* A infestés; de plus, dans les groupes infestés ayant un nombre moyen d'oocystes inférieur à 10, une forte proportion de moustiques (jusqu'à 95%) ne renfermaient aucune plasmodie. Statistiquement, une infestation légère ne suffit donc pas à modifier le taux de survie de l'ensemble du groupe.

On n'a observé aucune différence significative entre les taux de survie des moustiques non infestés et infestés pendant les 8 premiers jours d'incubation extrinsèque; néanmoins, du 9^{e} jour au 30^{e} jour de l'étude, le taux de survie moyen des groupes infestés ayant un nombre moyen d'oocystes $\geqslant 41$ différait significativement de celui des groupes non infestés. Au-delà de 30 jours, il n'y avait plus aucune différence significative entre les taux de survie moyens des deux groupes, mais cela peut simplement refléter le taux d'infestation des moustiques ayant survécu jusque là.

Le taux de survie journalier cumulé des groupes de moustiques ayant un nombre moyen d'oocystes $\leqslant 40$, compris entre 41 et 70, ou $\geqslant 71$, diminuait à mésure que le taux d'infestation augmentait. Par exemple, dans les groupes ayant un nombre moyen d'oocystes $\leqslant 40$, le taux de survie journalier cumulé ne présentait pas de différence significative par rapport à celui des groupes non infestés; en revanche, pour les groupes ayant un nombre moyen d'oocystes $\geqslant 71$, la différence était significative ($P < 0,01$), de même dans le groupe des moustiques ayant un nombre moyen d'oocystes compris entre 41 et 70 ($P < 0,10$). Le temps nécessaire pour que 50% des moustiques d'un échantillon soient morts (TL_{50}) était respectivement, pour les groupes appariés non infestés et infestés ayant un nombre moyen d'oocystes $\leqslant 40$, compris entre 41 et 70 et $\geqslant 71$, de 42,5 et 40,5 jours; 40,5 et 30,5 jours; et 43,5 et 17,0 jours. Les taux de survie cumulés jusqu'au neuvième jour étaient les mêmes pour tous les groupes.

Les moustiques portant en moyenne au moins 71 oocystes présentaient fréquemment une forte contamination bactérienne, et une décomposition de l'intestin et des glandes salivaires, avec altération de la couleur de ces dernières. Ces modifications, manifestement liées à l'infestation plasmodique massive ont très probablement abaissé le taux de survie de ces moustiques.

Les résultats des études décrites ici montrent que la présence de *Plasmodium* abaisse les taux de survie d'*A. dirus* A. Dans la nature, où les insectes sont soumis à davantage de contraintes environnementales qu'au laboratoire, cet effet peut être plus prononcé. L'abaissement du taux de survie d'un vecteur a des répercussions épidémiologiques importantes, puisqu'il diminue le nombre de pontes par femelle.

Annex 1

Use of unbiased estimates in the analysis

In analysing the data, we used the regression coefficients for the effect produced by a given number of oocysts in mosquitos from infected group 1 to estimate the number of oocysts in mosquitos in infected group 3, since it was not always possible to obtain an accurate count of oocysts in the latter group. The regression coefficients obtained are therefore biased.

If β is the true regression coefficient, its expectation value is given by $\beta/(1+\lambda)$, where $\lambda = \sigma_e^2/\sigma_x^2$ is the ratio of the error variance in estimating X to the variance in the X values used in the regression (*12*): σ_e^2 was estimated as 146.78 (18 degrees of freedom) using triplicates of infected mosquitos. An unbiased estimate of σ_x^2 is given by the variance in the number of oocysts in infected group 1 ($s_x^2 = 1269.19$; 35 degrees of freedom). In this way λ can be estimated to be 0.1156.

An unbiased estimate of the regression coefficient (β) is given by $b = (1+\lambda)b'$; however, the standard error is complicated to calculate. As indicated by Kendall & Stuart (*15*), the variance of b is

$$V(b) = V_{(1+\lambda)}b'^2 + (1+\lambda)^2 V_{(b')}$$

while the variance of λ, i.e., the variance of the ratio s_e^2/s_x^2, is given by

$$V(s_e^2)/(s_x^2)^2 + (s_e^2)^2 V(s_x^2)/(s_x^2)^4.$$

The variance of an estimated variance, s^2, is $2(s^2)^2$/degree of freedom and is used to obtain $V(s_e^2)$ and $V(s_x^2)$. The variance of λ is 0.0022502, and using this value we can obtain the variance of the unbiased estimates of regressions on the number of oocysts in infected group 3.

The regression of the survival rate of infected groups of mosquitos on the mean number of oocysts is $-0.1668(1+0.1156) = -0.1861$ days (standard error = 0.0303). The regression of the difference in the survival rate between non-infected and infected mosquitos on the mean number of oocysts of infected mosquitos is $0.1875(1+0.1156) = +0.2092$ days (standard error = 0.0282). For use in epidemiological models, unbiased estimates are appropriate.

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